The American journal of pathology (2012.Dec) 181巻6号:2094～2104.

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Short title: Reversion of ductular metaplasia of hepatocytes

Supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan

(#13670204, #16590303, and #18590362)

Abstract

We previously demonstrated that mature rat hepatocytes transdifferentiate to bile ductular cells when cultured in a three-dimensional collagen-rich matrix. Here, we show that the phenotype of transdifferentiated hepatocytes can be reversed by modulating culture conditions. Spheroidal aggregates of hepatocytes were cultured within a collagen gel matrix in the presence of serum and tumor necrosis factor-α. Spheroids transformed into ductular structures composed of small cuboidal cells, lost the expression of hepatocytic markers, while aberrantly expressed bile ductular markers. The transdifferentiated cells were then retrieved from the gels, plated on Matrigel-coated surfaces, and cultured in serum-free media. Cells spontaneously formed spheroidal aggregates and recovered hepatocytic phenotype. While Dexamethasone (Dex), which suppressed the phosphorylation of ERK and Jun N-terminal kinase, facilitated the recovery, the combination with interleukin-6 or oncostatin M resulted in the recovery of HNF-4α protein expression and the typical hepatocytic morphology and a decrease in the expression of bile ductular markers. A cDNA microarray analysis revealed that the hepatocyte-specific mRNA expression profile was recovered in these cells. Our results demonstrate that hepatocytes are able to recover their phenotypes following bile ductular transdifferentiation, suggesting that hepatocytic and bile ductular phenotypes may be mutually reversible.

Introduction

The appearance of irregular bile ductular structures is frequently observed in various chronic liver diseases associated with fibrosis.[1, 2] This process is called the atypical ductular reaction, and it has been interpreted as a regenerative proliferation of so-called hepatic progenitor cells, which are believed to be present in the vicinity of the portal tract.[3, 4] However, this reaction might also be due to the ductular metaplasia of hepatocytes.[1] Supporting this hypothesis, the ductular reaction can be induced in the centrilobular area in rodent chronic liver injury models induced by CCl_4 or thioacetamide, where no bile ducts or hepatic progenitors exist (Nishikawa et al., unpublished observation; see Supplemental Figure S1 at http://ajp.amjpathol.org). Furthermore, in chronic congestion of the human liver, the appearance of ductular hepatocytes with bile duct-specific cytokeratin has been observed in the centrilobular area.[5] This type of ductular reaction has been recently described and interpreted as a result of dedifferentiation of mature hepatocytes due to hypoxia.[6] It is important to precisely understand the origin and pathogenesis of the atypical ductular reaction because the prevention of its progression should be beneficial to maintain the proper functioning of the liver if the reaction is due to ductular metaplasia of hepatocytes.

We previously reported that mature hepatocytes could transdifferentiate into bile ductular cells when embedded within a type I collagen gel matrix and cultured in the presence of epidermal growth factor (EGF) and insulin.[7, 8] This phenotypic change is characterized by a loss of hepatocytic differentiation markers and the expression of bile ductular markers, but it is not

associated with the re-expression of markers of hepatic progenitor cells, such as delta-like. Other investigators have also been reported similar findings regarding the ductular transdifferentiation of hepatocytes both in vitro and in vivo.[9-11] Recently, we found that tumor necrosis factor-α (TNF-α) significantly enhanced ductular transdifferentiation (Nishikawa et al., submitted for publication).

An important question to be addressed is the reversibility of the bile ductular transdifferentiation of hepatocytes. In our observation of CCl_4-induced liver fibrosis, the centrilobular ductular reaction could diminish following cessation of injurious stimuli (see Supplemental Figure S1 at http://ajp.amjpathol.org). If the process is reversible, it may be possible to recover the hepatocytic phenotype in some types of ductular reactions, providing a rationale for the development of a new therapeutic approach targeted to ductular reaction in chronic liver diseases. In preliminary experiments, we retrieved the transdifferentiated hepatocytes from a collagen gel matrix and plated them on Matrigel, a basement membrane matrix that is known to be the most suitable for the maintenance of hepatocytic differentiation in vitro [12]. When cultured in serum-free medium, the hepatocytes gradually recovered their expression of albumin and tyrosine aminotransferase (TAT) mRNA. Although our data indicated that the transdifferentiation process may be reversible, the degree of redifferentiation was low, and the cells remained small and morphologically similar to ductular cells rather than to mature hepatocytes.

In this study, we attempted to find the condition under which transdifferentiated hepatocytes recovered mature phenotypes. Our previous experiments showed that interleukin (IL)-6 inhibited the loss of albumin expression in hepatocytes in the collagen gel matrix. Furthermore, the combination of Dex and oncostatin M (OSM), an IL-6 family cytokine, has been shown to be important for the maturation of mice hepatoblasts in vitro.[13, 14] Thus, we studied the effect of Dex and various cytokines on the recovery of hepatocytic phenotypes in our in vitro model, and found that both OSM and IL-6 could cause the mature hepatocytic phenotypes if used in combination with Dex. Our results indicate that the ductular transdifferentiation of hepatocytes is reversible and provide insights into the mutual phenotypic plasticity between hepatocytes and bile ductular cells.

Materials and methods

Chronic liver injury induced by CCl_4

Chronic liver injury induced by CCl_4 is characterized by centrilobular fibrosis with ductular reaction. To examine the reversibility of ductular reaction, a 1:1 mixture of CCl_4 and olive oil was administered to male F344 rats (7 weeks old) by gavage at a dose of 2 ml/kg twice per week for 8 weeks, followed by 3 weeks of recovery. The animals were sacrificed, and the liver tissues were fixed in phosphate-buffered 10% formalin, embedded in paraffin, and sectioned.

Isolation of rat hepatocytes

Hepatocytes were isolated from male Alb-DsRed2 transgenic rat livers (10-20 weeks old) by the collagenase perfusion method. In situ two-step collagenase liver perfusion and cell isolation were performed as previously described.[7, 8] Alb-DsRed2 rats, which express DsRed2 fluorescence under the control of the albumin enhancer/promoter, were used to monitor the level of albumin expression in hepatocytes.[15] The protocols for animal experimentation were approved by the Animal Research Committee, Akita University. All animal experiments adhered to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

Flow cytometric analysis

To examine whether the isolated hepatocyte fraction contain liver stem cell or progenitor cells, we performed flow cytometric analysis for several markers of liver stem cells. Isolated hepatocytes were fixed with phosphate-buffered 2% paraformaldehyde and then with 90% methanol. Fetal liver cells, separated from E17.5 livers by gentle mixing in collagenase solution, were used as positive controls. Cells were incubated with primary antibodies (1:400 dilution in phosphate-buffered saline with 2% fetal bovine serum [FBS-PBS]) for 1 hour at 4°C. The cells were washed three times in FBS-PBS and incubated with FITC-conjugated anti-rabbit IgG antibody (1:400 dilution) for 30 minutes. The cells were washed and immediately analyzed with a FACScan (Becton Dickinson). The primary antibodies used were

anti-α-fetoprotein (Proteintech, Chicago, IL), anti-DLK1 (Proteintech, Chicago, IL), anti-EpCAM (Proteintech, Chicago, IL), and anti-Thy1 (Novus Biologicals).

Three-dimensional collagen gel culture of rat hepatocytes

Isolated hepatocytes were plated onto positively charged plastic dishes (Primaria, Becton-Dickinson, NJ) to form spheroidal aggregates. Cells were cultured in serum-free Williams' Medium E supplemented with 10 mM nicotinamide, 10 ng/ml EGF (Roche Diagnostics, Mannheim, Germany), 10^{-7} M insulin (Sigma-Aldrich), 4.2 mM $NaHCO_3$, and penicillin/streptomycin (100 IU/ml and 100 μg/ml). After 2 or 3 days, spheroidal aggregates were harvested and used for three-dimensional cultures.

Three-dimensional cultures of hepatocytic spheroids within the collagen gel matrix were performed using a type I collagen solution (Cellmatrix type I-A; Nitta Gelatin, Japan) as described previously.[7] After being embedded within the gel, spheroidal aggregates were cultured in Williams' Medium E supplemented with 10 mM nicotinamide, 10% FBS, 10^{-7} M insulin, 10 ng/ml EGF, and 10 ng/ml TNF-α (R&D Systems, Minneapolis, MN).

To examine the reversibility of transdifferentiation, the ductular cells induced within the collagen gel were isolated by brief treatment with collagenase, transferred to multi-well plates coated with Matrigel (Becton-Dickinson, NJ), and cultured in serum-free Williams' Medium E supplemented with 10 mM nicotinamide, 10 ng/ml EGF, and 10^{-7} M insulin. Dex (1 μM;

Sigma-Aldrich, MO) and 10 ng/ml of various cytokines (IL-6, IL-1α, IL-1β, and oncostatin M; R&D Systems, Inc.) were added to the medium. In some experiments, a specific inhibitor of JNK (SP600125; 20 μM; Calbiochem, San Diego, CA) or MEK (PD98059; 20 μM; Calbiochem) was added to the medium to examine the effect of the suppression of these signaling pathways.

DsRed2 fluorescence intensity, which parallels albumin expression, was monitored with a fluorescence plate reader (Cytofluor, PerSeptive Biosystems, Framingham, MA). The DsRed2 signal was excited at 530 nm, and emission was detected at 620 nm.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA from freshly isolated and cultured hepatocytes was extracted using TRIzol reagent (Life Technologies Japan, Tokyo, Japan). Quantitative RT-PCR was performed by the ΔΔCt method using a QuantiTect SYBR Green RT-PCR Kit (Qiagen, Inc., Chatsworth, CA) and an Applied Biosystems StepOnePlus real-time PCR system (Life Technologies Japan). After reverse transcription at 50°C for 30 minutes and subsequent activation of HotStarTaq DNA polymerase at 95°C for 15 minutes, the cDNA was amplified with 40 cycles of a three-step PCR (94°C for 15 seconds, 60°C for 30 seconds, 72°C for 30 seconds). The specific primers were purchased from Qiagen (QuantiTect Primer Assays, Qiagen; albumin [Alb], QT00189679; TAT, QT00182308; glucose 6-phosphatase [G6Pase], QT00185948; phosphoenolpyruvate carboxykinase [PEPCK], QT01619975; bile salt export pump [BSEP],

QT00386008, cytokeratin [CK] 19, QT00376887; sex determining region Y-box [Sox9], QT00427602; osteopontin [OPN], QT00199101; secretin receptor [SCTR], QT00188426; cystic fibrosis transmembrane conductance regulator [CFTR], QT01612289; glyceraldehyde 3-phosphate dehydrogenase [GAPDH], QT00199633). The sequences of primers for HNF-4α were as follows: forward, 5'-GAAAATGTGCAGGTGTTGACCA-3'; reverse, 5'-AGCTTGAGGCTCCGTAGTGTTT-3'. The mRNA levels were normalized using GAPDH as a housekeeping gene.

Western blot analysis

Cells cultured within the collagen gel and Matrigel were retrieved by a brief incubation with collagenase and the Cell Recovery Solution (Becton-Dickinson, NJ), respectively, and lysed in RIPA buffer. Protein samples (30 μg of protein per lane) were subjected to SDS-PAGE using 10% polyacrylamide gels and were analyzed as described previously.[7] The primary antibodies used were anti-albumin (Nordic Immunological Laboratories, Tilburg, Netherlands), anti-CK19 (a gift from Dr. Atsushi Miyajima, Institute of Molecular and Cellular Biosciences, the University of Tokyo), anti-TAT (EPR6121, Novus Biologicals, Littleton, CO), anti-Sox9 (AB5535, Millipore Corporation, Billerica, MA), anti-HNF-4α (K9218, Perseus Proteomics, Tokyo, Japan), anti-HNF-1 (sc-8986, Santa Cruz Biotechnology, CA), anti-CCAAT/enhancer-binding protein (C/EBP) α (sc-61, Santa Cruz), anti-C/EBPβ (sc-150, Santa Cruz), anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (#9101, Cell Signaling Technology), anti-p44/42 MAPK (ERK1/2) (#9102, Cell Signaling Technology),

anti-phospho-SAPK/JNK (Thr183/Tyr185) (#9251, Cell Signaling Technology), anti-SAPK/JNK (#9258, Cell Signaling Technology), and anti-β-actin (Novus Biologicals).

Histochemical and morphological examination

Cultured cells and liver tissues were fixed in 10% formaldehyde in 0.1 M phosphate-buffered saline (pH 7.4), embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (HE) stain. Periodic acid-Schiff stain (PAS), PAS with diastase digestion (D-PAS), and sirius red staining were also performed in some experiments. Immunocytochemical or immunohistochemical staining was performed on deparaffinized sections treated with Target Retrieval Solution (DAKO Japan, Tokyo, Japan) using an anti-HNF-4α antibody (K9218, Perseus Proteomics) , anti-glutamine synthetase (ab73593, Abcam, Cambridge, UK), and anti-CK19 (a gift of Dr. Atsushi Miyajima, Institute of Molecular and Cellular Biosciences, the University of Tokyo, Tokyo, Japan) and Histofine-Max-PO for rat tissues (Nichirei Biosciences, Tokyo, Japan). In double staining with anti-glutamine synthetase antibody and anti-CK19 antibody, Histogreen (Linaris Biologische Produkte, Dossenheim, Germany) and 3,3’-diaminobenzidine were used as peroxidase chromogens. Immunofluorescence staining for Thy1 (CD90) (Novus Biologicals) was performed on smears of mature hepatocytes and fetal liver cells (E17.5) fixed by phosphate-buffered 4% paraformaldehyde. Antibody binding was detected with FITC-labeled anti-rabbit antibody (DAKO). For the observation of the ultrastructures, cells were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide and embedded in Epon resin. Ultrathin sections

were stained with uranylate and lead, and observed under an electron microscope (JEOL, Tokyo, Japan).

cDNA Microarray Analysis

We compared the gene expression profiles of transdifferentiated and redifferentiating cells using the 4 x 44 K Whole Rat Genome Oligo Microarray (Agilent Technologies Japan, Tokyo, Japan). Total RNA was extracted from freshly isolated hepatocytes, transdifferentiated hepatocytes cultured within the collagen gel for 14 days in the presence of TNF-α, cells cultured on Matrigel-coated surfaces for 7 days in the presence of IL-6 and Dex, and cells cultured on Matrigel-coated surfaces for 7 days in the presence of OSM and Dex. The gene expression profile of the transdifferentiated ductular cells was compared with that of the other samples, the genes that were upregulated more than 5-fold or were downregulated less than 0.2-fold were extracted, and the gene ontology (GO) category analysis and the hierarchical clustering were performed.

Intrahepatic transplantation of transdifferentiated hepatocytes

To examine the reversibility of transdifferentiation in vivo, fully transdifferentiated ductular cells, which were cultured within the collagen gel for 14 days in the presence of TNF-α, were transplanted into normal Wistar rat livers. Approximately $1x10^7$ cells were resuspended in 0.5 ml of phosphate-buffered saline and injected via a mesenteric vein. After 7 or 10 days, the animals were sacrificed and the livers were perfused with phosphate-buffered 4%

paraformaldehyde. Frozen sections were stained with Hoechst 33342 and observed by fluorescence microscopy. We performed two independent experiments.

Results

Phenotypic transition of adult hepatocytes into bile ductular cells within a collagen gel matrix in the presence of TNF-α

The spheroidal aggregates of Alb-DsRed2 hepatocytes emitted strong DsRed2 fluorescence that gradually decreased after being embedded within a collagen gel matrix, and the loss of fluorescent intensity was more rapid and profound when TNF-α was present in the medium (Figure 1A, B). The loss of fluorescence was accompanied by a prominent branching morphogenesis (Figure 1A) and an increase in proliferating cell nuclear antigen (Nishikawa et al, submitted for publication), but not by cell depletion due to cell death (see Supplemental Figure S2 at http://ajp.amjpathol.org). Quantitative RT-PCR analyses demonstrated that the expression of the hepatocytic differentiation marker genes (albumin, HNF-4α, TAT, G6Pase, PEPCK, and BSEP) rapidly decreased during the period of spheroid culture and that they were virtually lost at the end of collagen gel culture (Figure 1C). In contrast, the expression of bile duct/ductular marker genes (CK 19, Sox9, and OPN), which was completely absent in freshly isolated hepatocytes, increased during collagen gel culture (Figure 1C). The transdifferentiated hepatocytes expressed secretin receptor (SCTR) mRNA at high levels and cystic fibrosis transmembrane conductance regulator (CFTR) mRNA at low levels (see Supplemental Figure

S3 at http://ajp.amjpathol.org), indicating that they could exhibit some of the bile duct or cholangiocyte phenotype, although their morphology was more similar to bile ductular cells. Flow cytometric and immunofluorescence analyses confirmed that cells expressing putative liver stem cell markers were virtually absent in the initial hepatocytic fraction (see Supplemental Figures S4, 5 at http://ajp.amjpathol.org).

Recovery of hepatocytic phenotypes in transdifferentiated ductular cells after transfer to Matrigel-coated surfaces and the effects of Dex and various cytokines

Transdifferentiated ductular cells in the presence of TNF-α were retrieved from the collagen gel matrix by collagenase treatment and were cultured on Matrigel-coated surfaces under serum-free conditions. The cells spontaneously formed spheroid aggregates and gradually recovered DsRed2 fluorescence, and this recovery was facilitated by Dex (Figure 2A, B). Although the cytokines examined did not have significant effects on fluorescence recovery if applied alone, IL-6 and OSM remarkably enhanced the recovery synergistically with Dex (Figure 2A, B).

The recovery of fluorescence on Matrigel was associated with the recovery of the expression of hepatocytic differentiation marker mRNA (Figure 2C). Dex increased the expression of albumin, HNF-4α, TAT, G6Pase, and PEPCK but not BSEP (Figure 2C). Although IL-6 and OSM alone only slightly inhibited the expression of hepatocytic differentiation markers, they stimulated the expression of TAT and BSEP mRNA when added together with Dex (Figure

2C). In contrast, the mRNA expression of bile ductular differentiation markers decreased in the presence of Dex, and this suppression was significantly augmented by IL-6 or OSM (Figure 2C). Western blot analysis demonstrated that Dex, especially when applied in combination with IL-6 or OSM, enhanced the protein expression of hepatocytic differentiation markers, while inhibited bile ductular differentiation markers (see Supplemental Figure S6 at http://ajp.amjpathol.org).

We next examined the protein expression of transcription factors that are known to be involved in hepatocytic differentiation. Whereas freshly isolated hepatocytes expressed HNF-4α, HNF-1α, and C/EBPα at high levels, this expression dramatically declined after the initiation of collagen gel culture and was almost extinguished at the end of the culture (Figure 3A). Interestingly, after being transferred onto Matrigel-coated surfaces, HNF-4α and HNF-1α expression was recovered, particularly when IL-6 or OSM was added together with Dex (Figure 3A; for HNF-4α, also see Supplemental Figure S6 at http://ajp.amjpathol.org). Although C/EBPα expression slightly increased after the transfer, Dex inhibited this expression (Figure 3A). C/EBPβ expression, which was low in hepatocytes and increased during the culture period, was inhibited by Dex but slightly enhanced by IL-6 and OSM (Figure 3A).

Because these results suggested that HNF-4α protein expression was closely associated with the recovery of hepatocytic phenotypes, we performed immunocytochemistry to detect HNF-4α. Whereas freshly isolated hepatocytes strongly expressed HNF-4α protein in their

nuclei, ductular cells induced by culturing within the collagen gel matrix with TNF-α completely lost this expression (Figure 3B). After transfer to Matrigel-coated surfaces, only a few nuclei became positive for HNF-4α in control cells (Figure 3B). Whereas Dex alone slightly increased the number of HNF-4α expressing cells, this effect was markedly enhanced by the presence of IL-6 or OSM (Figure 3B).

Recovery of hepatocytic phenotypes by Dex is associated with the inhibition of MEK and JNK pathways

To explore the possible mechanisms of the recovery of hepatocytic phenotypes, we examined the phosphorylation status of several signaling proteins. ERK phosphorylation increased in culture and was enhanced by IL-6, OSM, and TNF-α, but was strongly inhibited by Dex (Figure 4A). Although JNK2/3 was transiently phosphorylated in freshly isolated hepatocytes, which could be explained by cell stress during the isolation steps (Figure 4A). Although JNK2/3 was again phosphorylated in collagen gel culture with TNF-α, its phosphorylation level was decreased after transfer to Matrigel-coated surfaces, especially in the presence of Dex (Figure 4A). JNK1 was only slightly phosphorylated in freshly isolated hepatocytes, but it was markedly phosphorylated in collagen gel culture with TNF-α (Figure 4A). Although the phosphorylation level of JNK1 was reduced after transfer, it was enhanced by IL-6, OSM, and TNF-α (Figure 4A). Dex efficiently repressed the effect of these cytokines, apparently through the inhibition of JNK protein expression (Figure 4A).

Because the inhibition of the MEK-ERK and JNK-c-Jun pathways may be associated with the recovery of hepatocytic phenotypes, we examined whether a MEK inhibitor (PD98059) or a JNK inhibitor (SP600125) affected this recovery. These inhibitors indeed augmented the recovery of DsRed2 fluorescence after transfer to Matrigel-coated surfaces, and the effect of the inhibitors was more prominent than that of Dex alone (Figure 4B). However, the effects of the inhibitors on HNF-4α expression levels and cell morphology were comparable to those by Dex alone (Figure 4C). While PD98059 slightly enhanced the recovery of albumin expression, these inhibitors did not significantly affect the protein expression levels of hepatocytic and bile ductular differentiation markers (see Supplemental Figure S6 at http://ajp.amjpathol.org).

IL-6+Dex or OSM+Dex restores mature hepatocytic morphology after ductular transdifferentiation of hepatocytes

Mature hepatocytes have one or two large nuclei with a prominent nucleolus and abundant eosinophilic cytoplasm, whereas interlobular bile ducts and bile ductules are composed of small cuboidal epithelial cells (Figure 5A). After being cultured within the collagen gel matrix with TNF-α for 14 days, hepatocytes transformed into small cuboidal cells forming small tubular structures, morphologically similar to bile ductules (Figure 5B). Although collagenase-digested and dispersed ductular cells formed aggregates on Matrigel-coated surfaces and recovered some hepatocytic markers, they were small, polygonal, and distinct from the original mature hepatocytes (Figure 5C). The addition of Dex, IL-6, or OSM alone did not affect the morphology (Figure 5D, E). However, when IL-6 or OSM was added

together with Dex, the majority of the cells was enlarged and exhibited mature hepatocytic features, including large nuclei, prominent nucleoli, and abundant eosinophilic cytoplasm (Figure 5F, G). Furthermore, some of these cells accumulated glycogen, which was demonstrated by PAS and D-PAS staining (Figure 5H, I).

The ultrastructural analysis of the branching ductular structures formed within the collagen gel matrix revealed that they were surrounded by basement membranes and possessed sparse microvilli on the luminal surfaces (Figure 6A, B). In contrast, the redifferentiating cells on the Matrigel had microvilli on their surfaces, lacked basement membranes (Figure 6C), and had an extensive cytoplasm that contained many mitochondria, rough endoplasmic reticula, and a few lipid droplets (Figure 6D). Furthermore, they formed bile canaliculus-like lumens with well-developed cell adhesion complexes (Figure 6E, F).

The mRNA expression profile of redifferentiating hepatocytes treated with IL-6+Dex and OSM+Dex on Matrigel is similar to that of freshly isolated hepatocytes

To analyze the level of redifferentiation into hepatocytes after transfer to Matrigel, we analyzed the mRNA extracted from freshly isolated hepatocytes, transdifferentiated hepatocytes within the collagen gel, and redifferentiating hepatocytes on Matrigel in the presence of IL-6+Dex or OSM+Dex by a cDNA microarray. Gene ontology (GO) analysis revealed that the transdifferentiation process was associated with changes in the gene products of various GO terms, and interestingly, changes in the opposite directions were found in the redifferentiation

process (see Supplemental Table S1 at http://ajp.amjpathol.org). Genes that were upregulated more than 5-fold and were downregulated less than 0.2-fold were selected and analyzed. The amounts of differentially expressed mRNAs were 4849, 1762, and 1602 for freshly isolated hepatocytes, redifferentiating hepatocytes treated with IL-6+Dex, and those treated with OSM+Dex, respectively (Figure 7). A Venn diagram analysis identified 799 differentially expressed genes common to the three samples (Figure 7), and a hierarchical clustering analysis was performed for these genes. The heat map showed that the pattern in the transdifferentiated hepatocytes was quite different from that of freshly isolated hepatocytes, whereas the redifferentiating hepatocytes treated either by IL6+Dex or OSM+Dex generated patterns similar to that of freshly isolated hepatocytes (Figure 7). The Pearson's correlation coefficients of IL-6+Dex- vs. OSM+Dex-treated regenerating hepatocytes, IL-6+Dex-treated regenerating hepatocytes vs. freshly isolated hepatocytes, and IL-6+Dex-treated regenerating hepatocytes vs. transdifferentiated hepatocytes were 0.98, 0.75, and 0.24, respectively (Figure 7).

Evidence for reversion of transdifferentiated phenotype of hepatocytes in vivo

We previously demonstrated that altered phenotypes in 5 day-cultured hepatocytic spheroids were more efficiently reversed after intrahepatic transplantation as compared with intrasplenic transplantation.[16] To examine whether fully transdifferentiated hepatocytes could reverse their original phenotype in the normal liver environment, we transplanted transdifferentiated Alb-DsRed2 hepatocytes, which were cultured within the collagen gel matrix for 14 days in the presence of TNF-α, into intact livers of Wistar rats via the portal vein. After 7 or 10 days,

scattered large hepatocytes emitting DsRed2 fluorescence were reproducibly observed in the liver parenchyma (Figure 8), suggesting the reversible nature of ductular transdifferentiation in a normal liver microenvironment.

Discussion

In this study, we demonstrated that transdifferentiated hepatocytes in vitro could recover the original hepatocytic phenotypes if they were placed in a more physiological condition. The transdifferentiated cells retrieved from the collagen gel matrix spontaneously formed spheroidal aggregates on Matrigel-coated surfaces. In our preliminary experiments, cells plated with a serum-containing medium spread across the surface without forming spheroids, and there was no recovery of DsRed2 fluorescence. Cultures of hepatocytes in spheroidal aggregates have been shown to be beneficial in the maintenance of differentiated phenotypes of hepatocytes.[17, 18] Thus, spheroid formation appeared to be important for the recovery of hepatocytic phenotypes, probably via increasing cell-cell contacts in self-organized three-dimensional structures. Furthermore, the interaction of cells with the components of the Matrigel might play important roles in the recovery partly through the modulation of expression of transcription factors, including HNF-4α,[19] and the activation of integrin-linked kinase that is involved in transmitting integrin signaling to the interior of the cell.[20, 21]

The recovery of albumin expression in transdifferentiated hepatocytes was enhanced by Dex, which has been shown to increase albumin and TAT expression in spheroidal aggregate

cultures of newborn rat hepatocytes[18] and decrease CK 19 mRNA expression in monolayer-cultured rat hepatocytes.[7] Although the mechanism of the effect of Dex on hepatocytic phenotypes has not been elucidated, this study suggests that the inhibition of the MEK and JNK signaling pathways may be important. In a previous study, we also found that PD98059 inhibited aberrant CK 19 expression in hepatocytes cultured within the collagen gel matrix.[7] Dex inhibits the phosphorylation of JNK via a direct interaction with the glucocorticoid receptor in rat hepatocytes.[22] However, interestingly, Dex decreased JNK at the protein level in our experiments, suggesting that this may be a long-term effect of Dex in the regulation of JNK activity.

Although the phenotypic reversion toward hepatocytes induced by Dex was limited, it was markedly enhanced by IL-6 and OSM, which belong to the IL-6 family of cytokines. Our results illustrate the differential effects of inflammatory cytokines on the regulation of differentiation between hepatocytes and bile ductules: IL-6 and OSM facilitate or maintain hepatocytic differentiation, whereas TNF-α induces the bile ductular transdifferentiation of hepatocytes. The in vitro maturation of fetal mouse hepatoblasts is accomplished by the addition of Dex with OSM, through the activation of gp130.[14] which is crucial for the maturation of hepatocytes. Furthermore, fetal mouse hepatoblasts are able to fully mature with the expression of tryptophan oxygenase in high-cell-density culture with Matrigel in the presence of Dex and OSM.[23] Interestingly, in contrast to the reversion of transdifferentiated hepatocytes, IL-6 is ineffective at inducing the differentiation of fetal hepatocytes toward

mature hepatocytes in vitro. Although OSM can directly bind to gp130 on the target cells and then form a complex with leukemia inhibitory factor receptor (LIFR), IL-6 needs to be bound to the IL-6 receptor, either the membrane-bound or soluble form, to associate with gp130.[24] Although IL-6 itself does not affect the proliferation of fetal rat hepatocytes, a chimera of IL-6 and its soluble receptor stimulates their proliferation and maturation.[25] IL-6 receptors may not express or function in fetal hepatocytes. Our results highlight both the similarities and differences between the maturation of fetal hepatocytes and the recovery of transdifferentiated hepatocytes.

One of the most impressive molecular events associated with the synergistic effects of Dex and IL-6 or OSM was the recovery of HNF-4α at the protein level. Although Dex alone enhanced HNF-4α mRNA levels, there was not a significant associated increase in HNF-4α protein levels, suggesting the involvement of posttranscriptional control mechanisms. HNF-4α protein levels can be modulated without a change in HNF-4α mRNA expression in HepG2 cells.[26, 27] HNF-4α is known to be one of the most important transcription factors that drive the differentiation of the hepatic lineage.[28] HNF-4α binding sites have been identified in many hepatocyte-specific genes that are involved in the metabolism of glucose, lipids, amino acids, and xenobiotics.[29, 30] A recent analysis employing a new integrated approach identified approximately 240 direct human target genes, including both known and unexpected targets.[31] Interestingly, the recovery of hepatocytic phenotypes induced by IL-6+Dex or OSM+Dex was also associated with a marked increase in the HNF-1α protein, another liver-specific transcription factor whose gene

expression is known to be directly controlled by HNF-4α.[29] The correlated expression of these two liver-specific transcription factors has been observed in the well-differentiated status of primary rat hepatocytes cultured on Matrigel.[32]

In our three-dimensional culture model, hepatocytic spheroids transformed into ductular structures composed of small cuboidal cells, which were morphologically similar to bile ductules. However, after redifferentiation on Matrigel in the presence of IL-6+Dex or OSM+Dex, they recovered the original morphology of mature hepatocytes with accumulated glycogen in their cytoplasm. The phenotypic recovery was further confirmed by cDNA microarray analysis, which demonstrated a significant reversal of the altered gene expression profiles that occurred after transdifferentiation. The mechanisms regulating the size of the hepatocytes are largely unknown. Although the phosphatidylinositol-3 kinase/Akt pathway has been implicated in the regulation of cell size[33] and has been shown to be responsible for hepatocyte hypertrophy during liver regeneration,[34] Akt phosphorylation increased during transdifferentiation (Nishikawa et al., submitted for publication). As suggested by experiments using conditional HNF-4α knockout mice, in which small hepatocytes with decreased glycogen accumulation were found in the fetal liver (E18.5) with abnormally formed sinusoidal structures.[35] the coordinated actions of molecular events induced by this transcription factor may be indispensable for the development, maintenance, and recovery of normal hepatocytic phenotype.

Our results suggest that hepatocytic and bile ductular phenotypes may be mutually reversible. While the present study mainly focused on the in vitro experiments, we demonstrated that fully transdifferentiated Alb-DsRed2 hepatocytes could recover their fluorescence and hepatocytic morphology when they settled in the normal liver environment. We also suggested that the centrilobular ductular reaction induced by chronic CCl_4 injury might be reversible. Although the origin of ductular cells in the ductular reaction is still debated and a recent work did not find evidence for formation of ductular cells from hepatocytes,[36] we have obtained clear evidence for the involvement of ductular transdifferentiation of mature hepatocytes in the ductular reaction, using several hepatocyte lineage tracing systems in mice (Nagahama et al., manuscript in preparation). Our hypothesis of phenotypic plasticity between hepatocytes and bile ductular cells in the adult liver may have relevance to the disease process, as well as the recovery, in chronic liver diseases.

Acknowledgments

We thank Eiji Kobayashi for generously providing the Alb-DsRed2 transgenic rats and Yasuharu Ueno for helpful suggestions regarding the genotyping of the transgenic rats. We appreciate the excellent technical assistance of Akiko Yagisawa, Reiko Ito, Makiko Kawamata, and Yoko Okada.

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Figure legends

Figure 1: Transdifferentiation of Alb-DsRed2 transgenic rat hepatocytes within a collagen gel matrix. (A) Phase-contrast microscopy and corresponding DsRed2 fluorescence of hepatocytic spheroids immediately after being embedded within a collagen gal matrix (0 d) and cultured for 14 days in the absence or presence of 10 ng/ml TNF-α. (B) The time course of DsRed2 fluorescence intensity of the hepatocytic spheroids in collagen gel culture (n=4). *P value < 0.01, compared with control. (C) Quantitative RT-PCR analysis of the expression of hepatocyte-specific (albumin, HNF-4α, TAT, G6Pase, PEPCK, BSEP) and bile duct/ductule-specific (CK 19, Sox9, OPN) genes in freshly isolated hepatocytes and in culture. Bars indicate the SD in a representative experiment performed in triplicate.

Figure 2: Recovery of hepatocytic phenotypes in the transdifferentiated hepatocytes after being transferred to Matrigel-coated surfaces. (A) Phase-contrast microscopy and corresponding DsRed2 fluorescence of cells retrieved from the collagen gel, transferred to Matrigel-coated surfaces, and cultured for 7 days in serum-free control medium or medium containing IL-6+Dex or OSM+Dex. (B) The time course of the recovery of DsRed2 fluorescence intensity of the transdifferentiated hepatocytes after being transferred to Matrigel-coated surfaces (n=4). *P value < 0.05, **P value < 0.01, compared with Dex (unpaired two-tailed t-test); $^{\#}$P value < 0.01, compared with control (unpaired two-tailed t-test). (C) Quantitative RT-PCR analysis of the expression of hepatocyte-specific (albumin, HNF-4α, TAT, G6Pase, PEPCK, BSEP) and bile duct/ductule-specific (CK 19, Sox9, OPN) genes in

transdifferentiated hepatocytes. Bars indicate the SD in a representative experiment performed in triplicate.

Figure 3: Recovery of the expression of hepatocyte-related transcription factors in the redifferentiating cells on Matrigel-coated surfaces. (A) Western blot analysis of expression of HNF-4α, HNF-1α, C/EBPα, and C/EBPβ. Protein was extracted from freshly isolated hepatocytes, spheroids (cultured for 2 days on Primaria dishes), transdifferentiated hepatocytes (cultured within a collagen gel matrix in the presence of TNF-α for 14 days), and redifferentiating cells (cultured on Matrigel-coated surfaces for 6 days in the absence [control] or presence of Dex, IL-6, OSM, IL-6+Dex, or OSM+Dex) and analyzed. (B) HNF-4α immunocytochemistry. Freshly isolated hepatocytes (Heps), transdifferentiated hepatocytes cultured within a collagen matrix in the presence of TNF-α for 14 days (In Gel [TNF-α]), and redifferentiating hepatocytes cultured for 6 days on Matrigel in the control medium (Control) or medium containing Dex, IL-6+Dex, or OSM+Dex. Scale bars, 20 μm.

Figure 4: Dex-induced suppression of the ERK and JNK phosphorylation in the redifferentiating cells on Matrigel-coated surfaces. (A) Western blot analysis of total and phosphorylated ERK and JNK. Protein was extracted from freshly isolated hepatocytes, spheroids (cultured for 2 days on Primaria dishes), transdifferentiated hepatocytes (cultured within a collagen gel matrix in the presence of TNF-α for 14 days), and redifferentiating cells (cultured on Matrigel-coated surfaces for 6 days in the absence [control] or presence of Dex,

IL-6, OSM, TNF-α, IL-6+Dex, or OSM+Dex) and analyzed. (B) The time course of DsRed2 fluorescence intensity of transdifferentiated hepatocytes on Matrigel-coated surfaces (n=4) comparing the control, Dex, PD98059 (MEK inhibitor), and SP600125 (JNK inhibitor). *P value < 0.05, **P value < 0.01, compared with control (unpaired two-tailed t-test). (C) HNF-4α immunocytochemistry of redifferentiating hepatocytes cultured on Matrigel-coated surfaces for 6 days in the presence of Dex, PD98059, or SP600125. Scale bars, 20 μm.

Figure 5: Mature hepatocytic morphology in redifferentiating hepatocytes induced by IL-6 and OSM in the presence of Dex. (A-G) HE staining, (H) PAS staining, (I) D-PAS staining. (A) Normal Alb-DsRed2 rat liver tissue. An interlobular bile duct (arrow) and two bile ductules (arrowheads) are seen. (B) Transdifferentiated hepatocytes induced by culturing for 14 days within a collagen gel matrix in the presence of TNF-α. (C-H) Redifferentiating hepatocytes cultured for 6 days on Matrigel-coated surfaces. (C) Control, (D) Dex, (E) IL-6, (F) IL-6+Dex, (G) OSM+Dex, (H, I) IL-6+Dex (arrows indicate two identical cells in adjacent sections without and with diastase treatment). Scale bars, 20 μm.

Figure 6: Appearance of mature hepatocytic ultrastructures in redifferentiating hepatocytes treated with IL-6 and Dex. (A, B) Transdifferentiated hepatocytes induced by culturing for 14 days within a collagen gel matrix in the presence of TNF-α. The arrowheads in (B) indicate basement membranes around the ductular structure. (C-F) Redifferentiating hepatocytes cultured for 6 days on Matrigel-coated surfaces in the presence of IL-6+Dex. The arrowheads

in (C) indicate the surface of the cell lacking basement membranes.

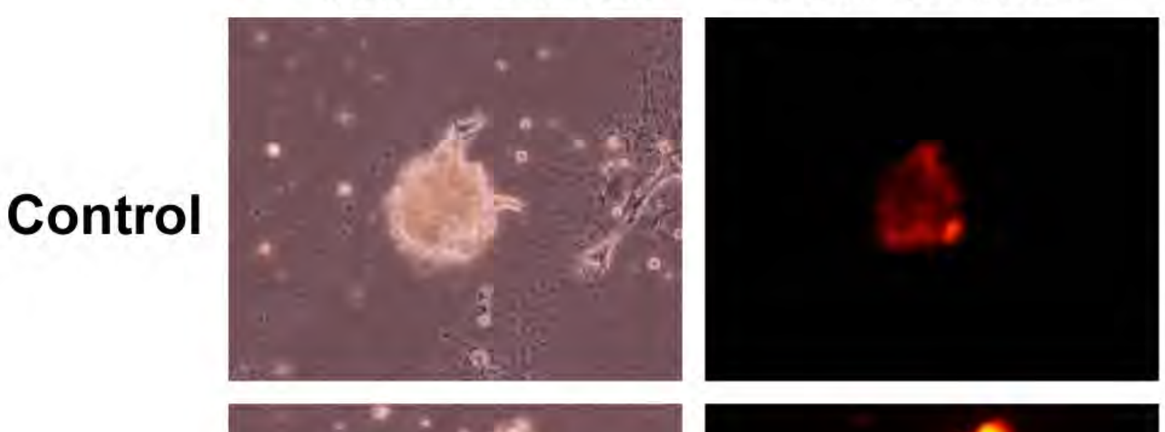
Figure 7: Recovery of the mRNA expression profile of mature hepatocytes by culturing on Matrigel-coated surfaces in the presence of IL-6+Dex or OSM+Dex. A cDNA microarray analysis of mRNA extracted from freshly isolated hepatocytes, transdifferentiated hepatocytes induced by culturing for 14 days within a collagen gel matrix in the presence of TNF-α, and redifferentiating hepatocytes for 6 days on Matrigel in the presence of IL-6+Dex or OSM+Dex. Differentially expressed genes common to the three samples (799 genes) were subject to a hierarchical clustering analysis. Pearson's correlation coefficients are indicated at the left of the heat map.

Figure 8: Evidence for reversibility of ductular transdifferentiation of hepatocytes in vivo. Fluorescence microscopy of rat livers 7 days after transplantation of transdifferentiated Alb-DsRed2 hepatocytes, which were cultured within the collagen gel for 14 days with TNF-α. Merged images of DsRed2 fluorescence and nuclear staining with Hoechst 33342 (A, B) or 4',6-diamidino-2-phenylindole (C, D) from two independent experiments. The arrow in (A) indicates DsRed2 fluorescent cells, which are enlarged in (B). Scale bars, 20 μm.

Figure 1 (Sone et al.)

Figure 2 (Sone et al.)

A



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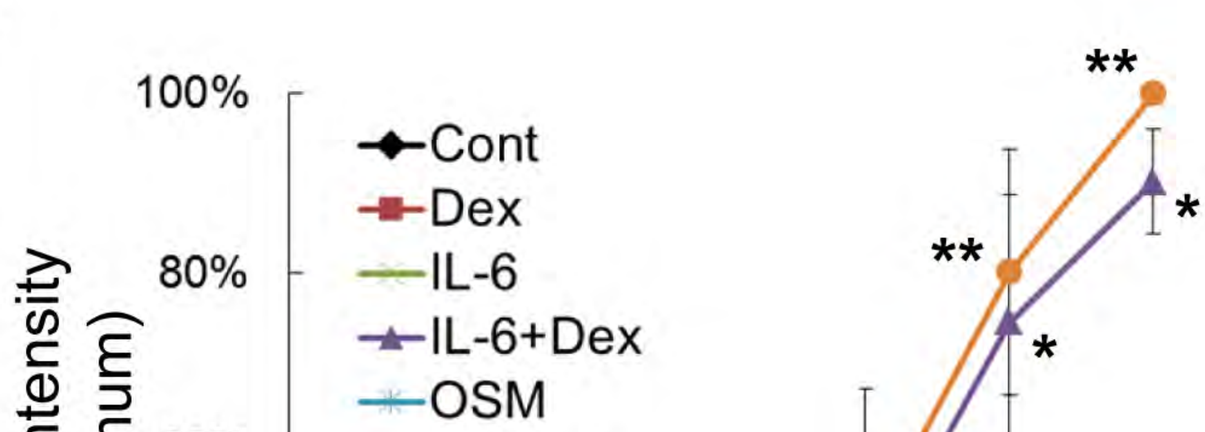

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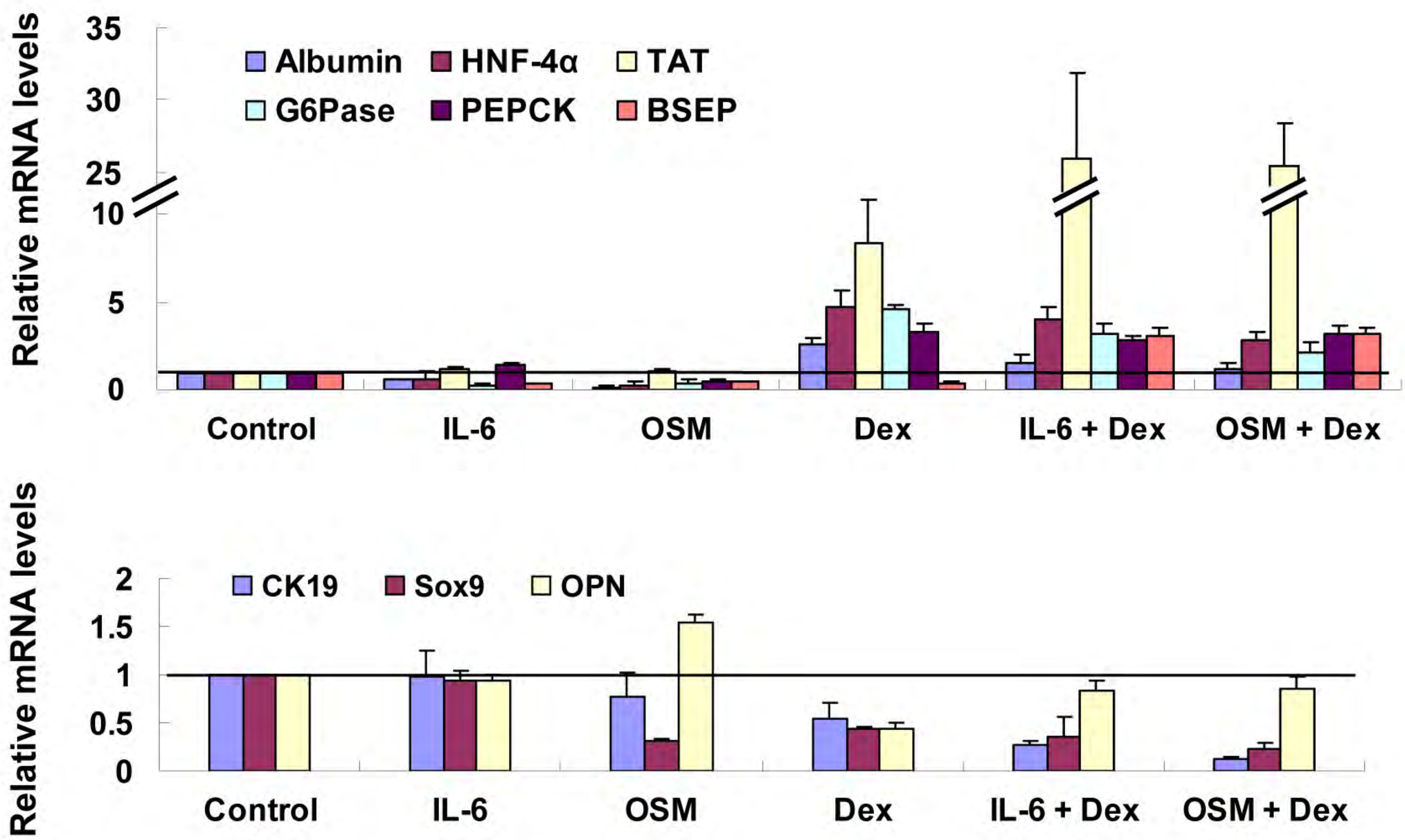
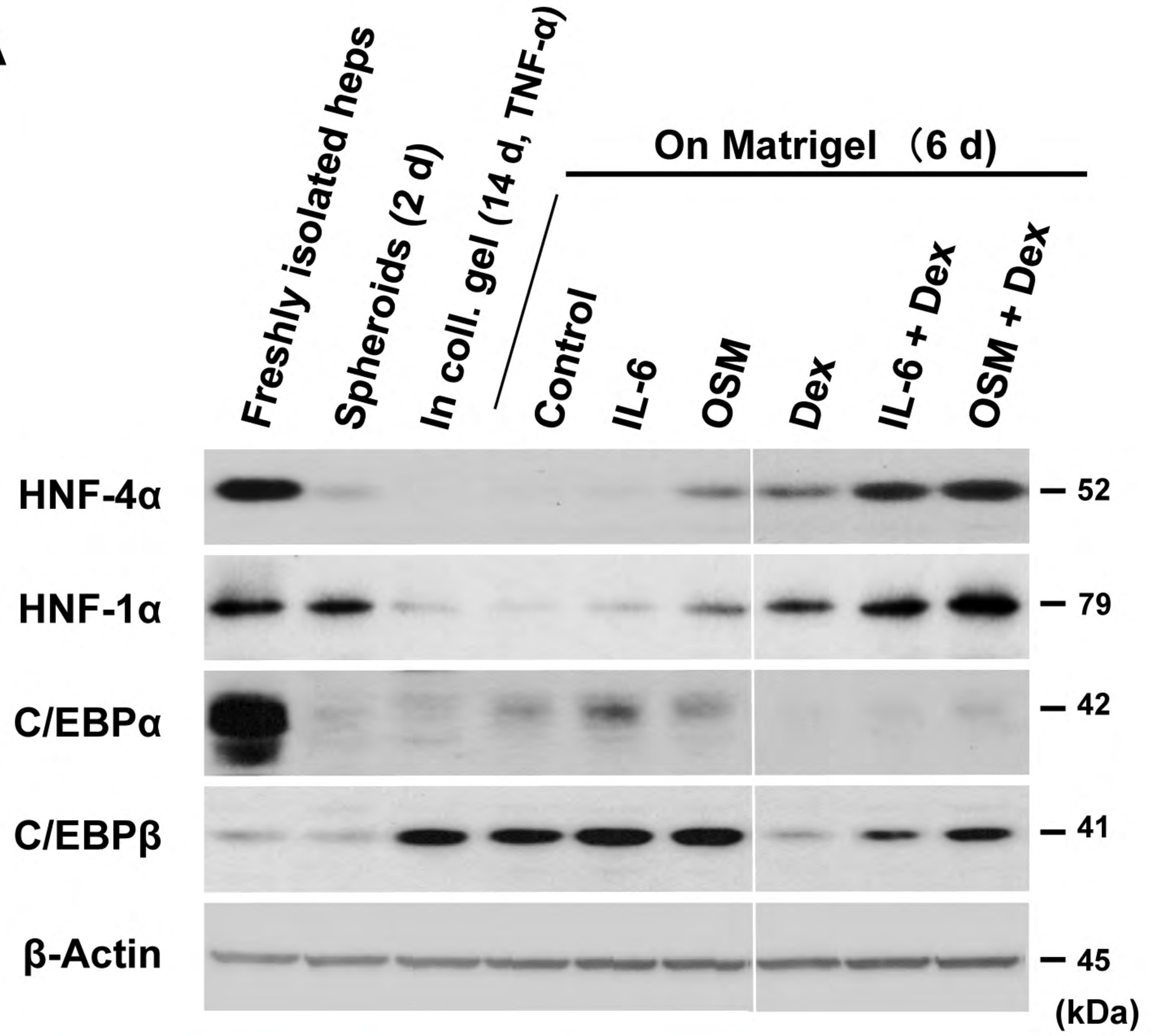


Figure 3 (Sone et al.)

A



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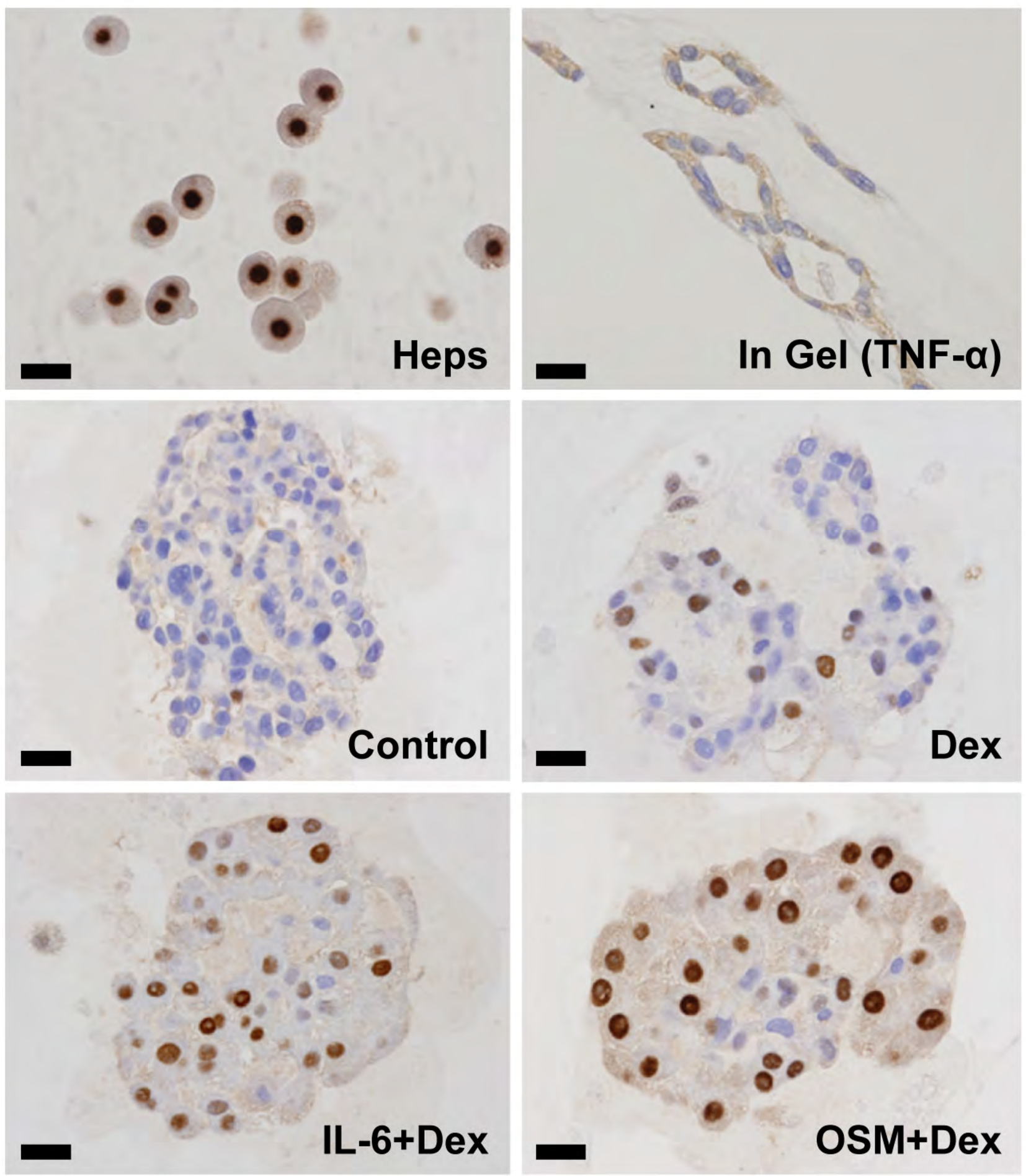
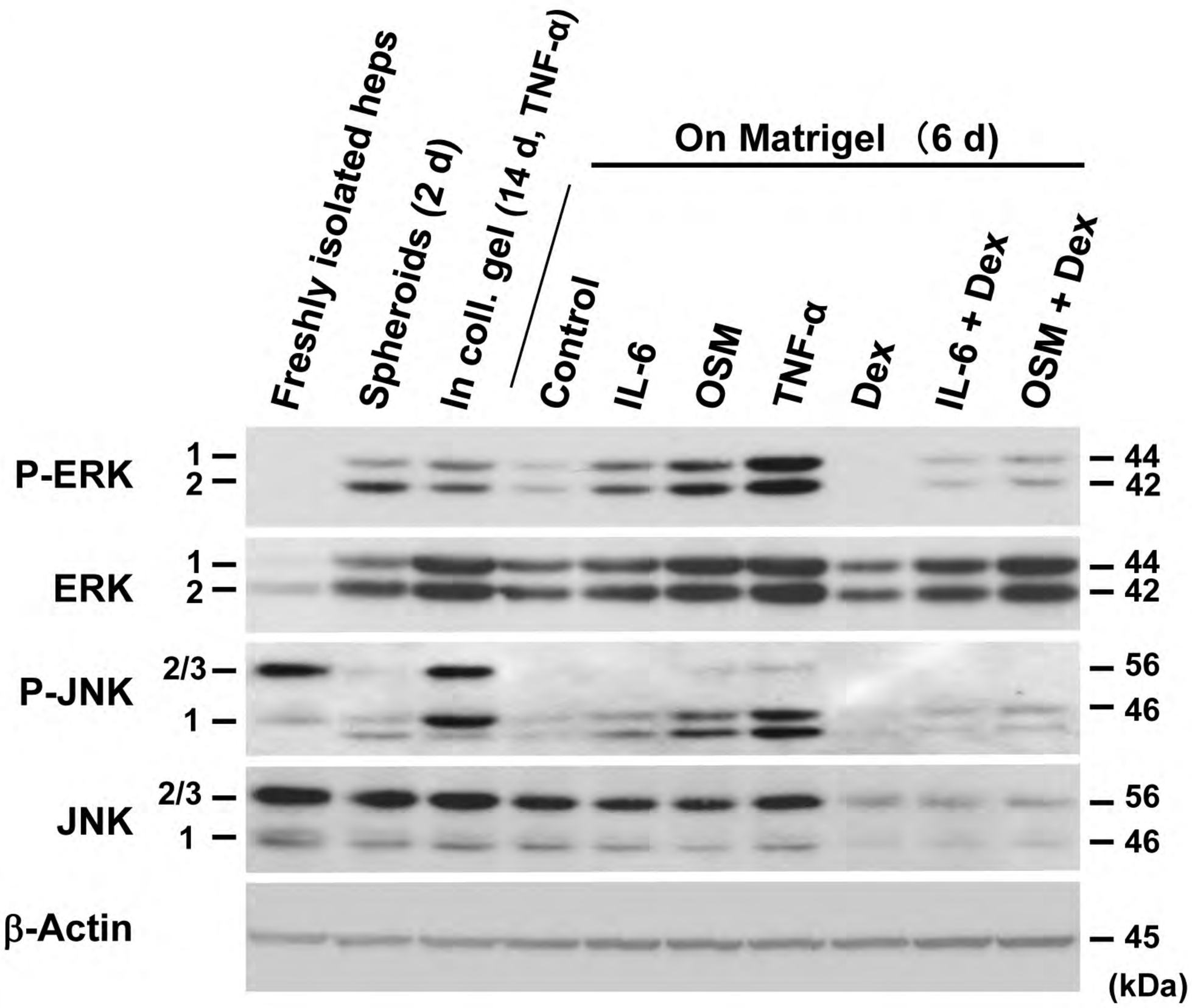


Figure 4 (Sone et al.)

A



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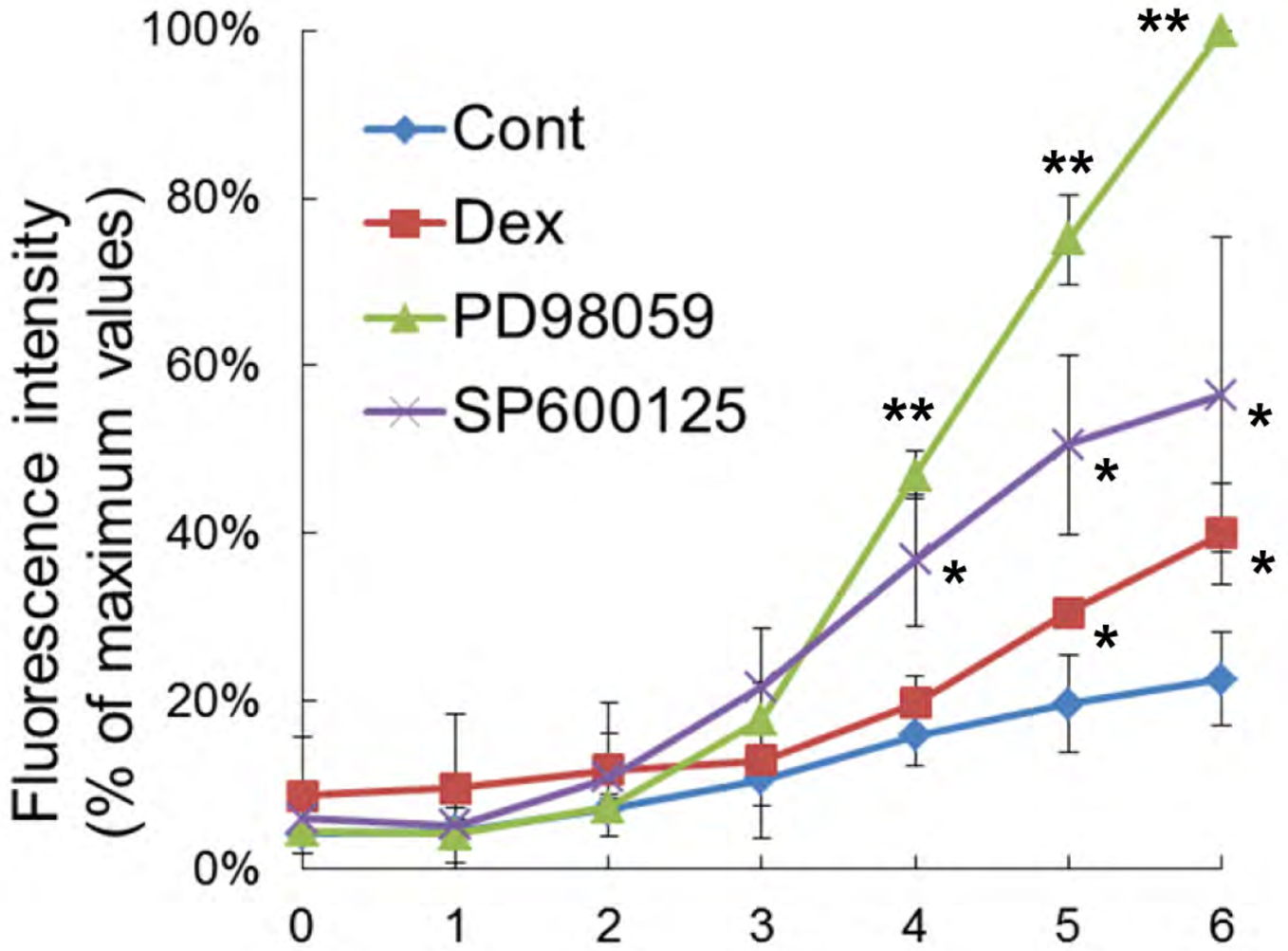

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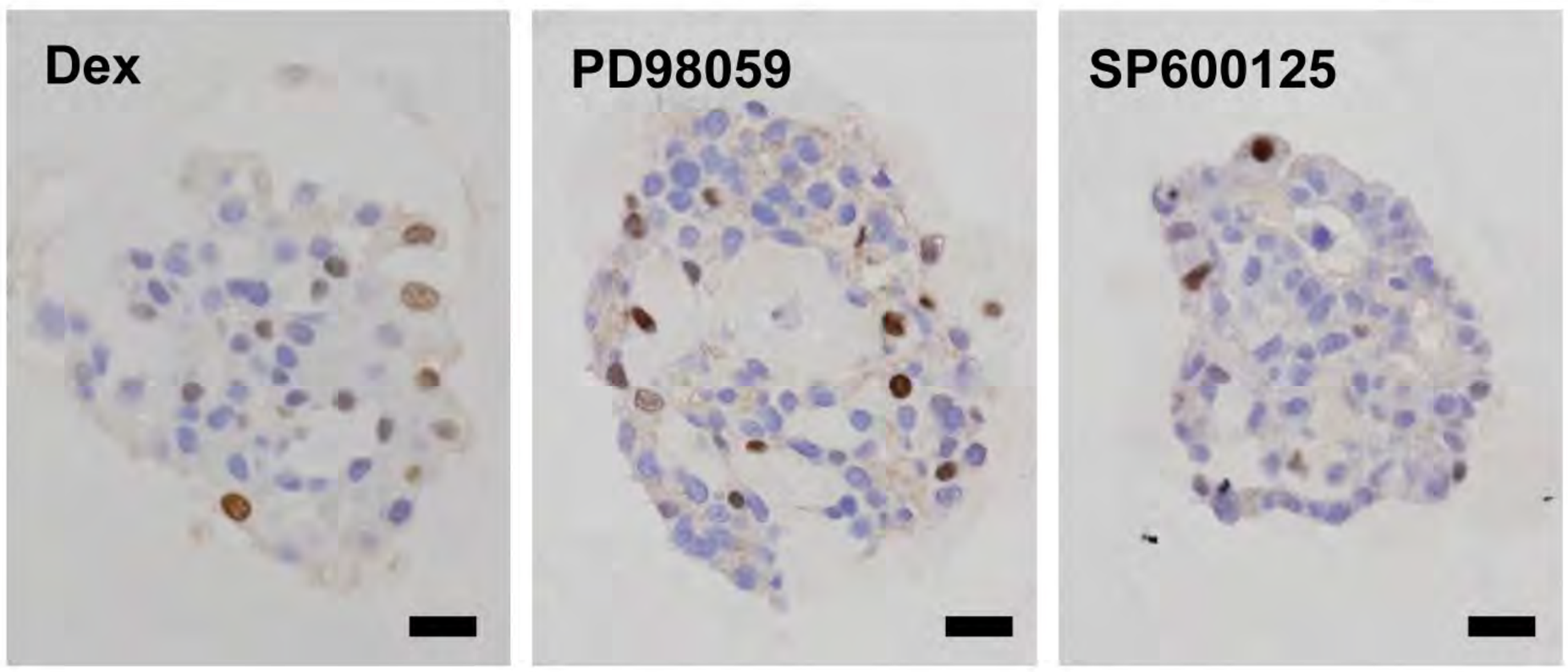
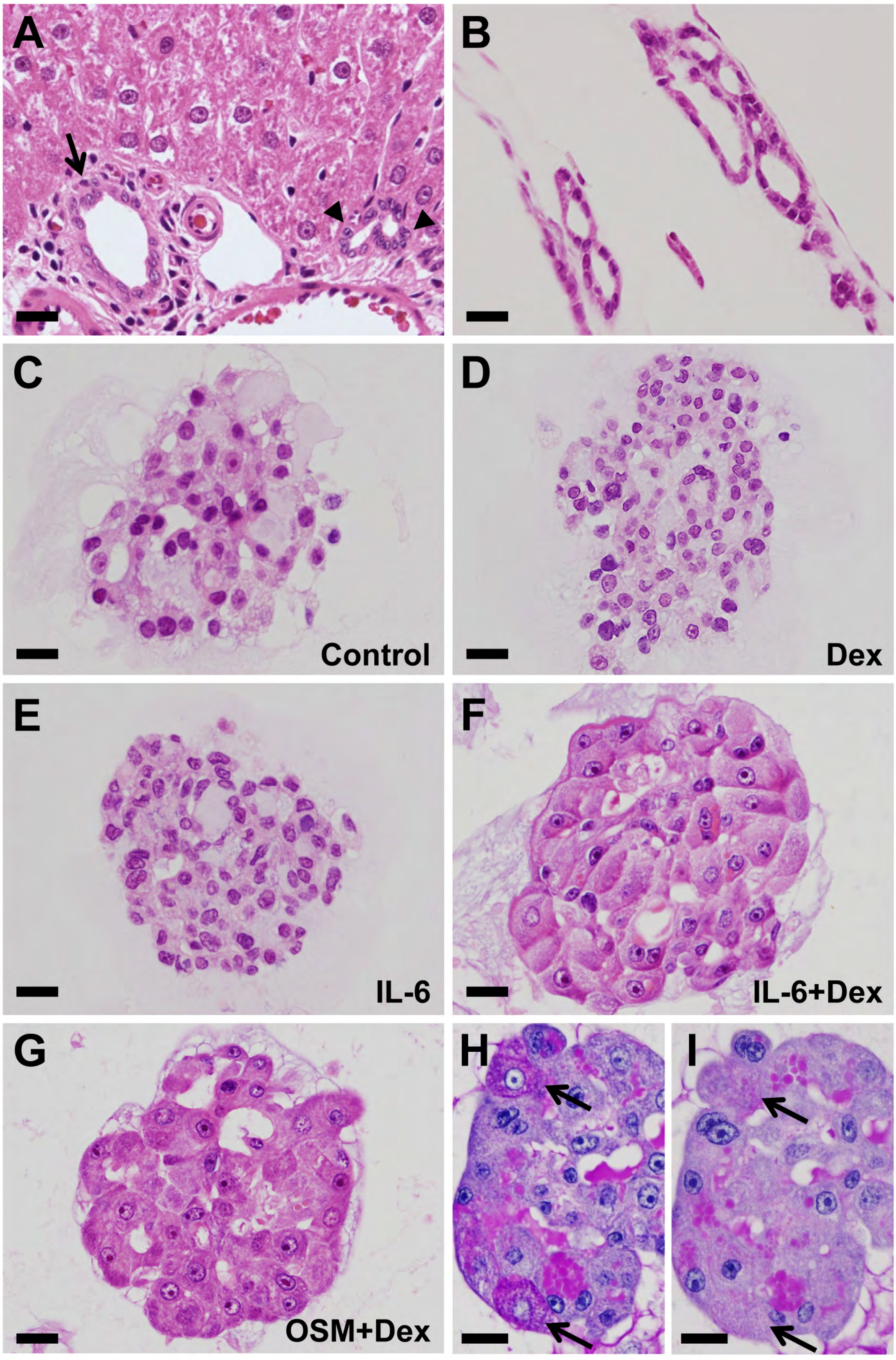


Figure 5 (Sone et al.)



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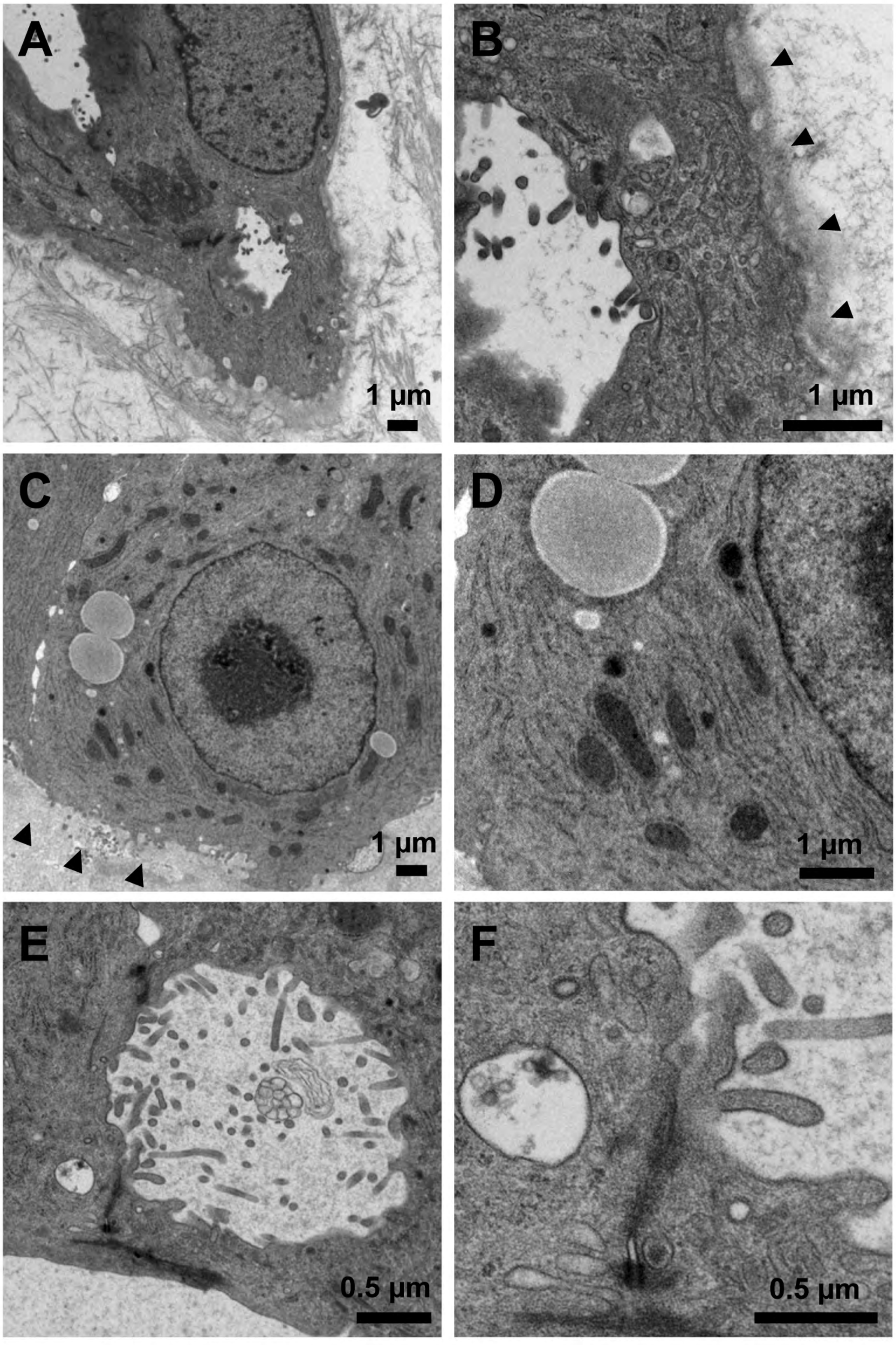

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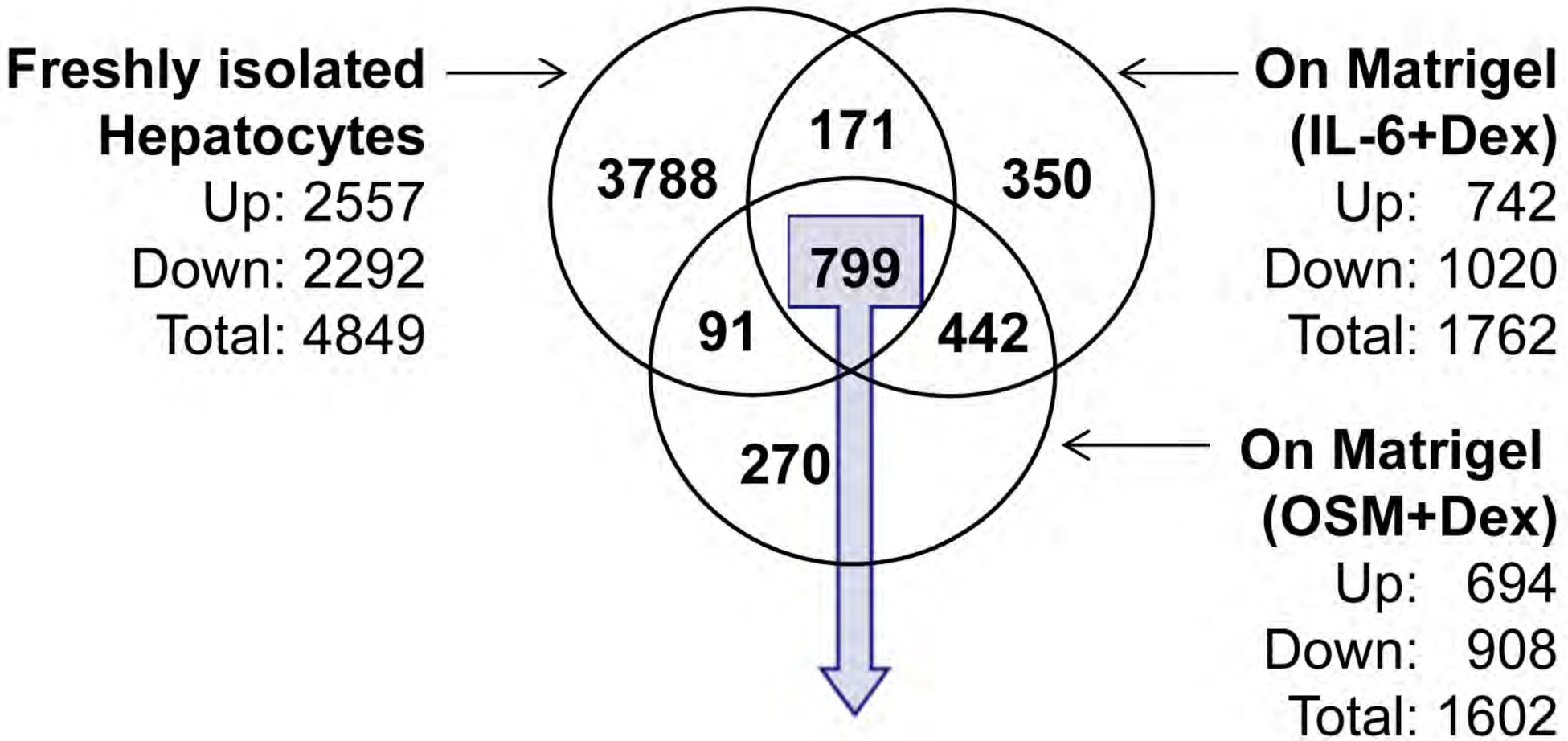

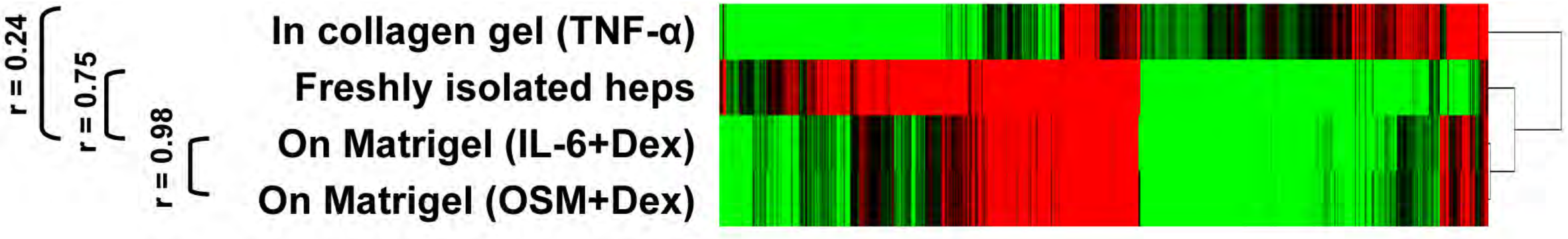

Figure 8 (Sone et al.)